SNAP-23 participates in SNARE complex assembly in rat adipose cells

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SNARE proteins are required for vesicle docking and fusion in eukaryotic cells in processes as diverse as homotypic membrane fusion and synaptic vesicle exocytosis [SNARE stands for SNAP receptor, where SNAP is soluble NSF attachment protein]. The SNARE proteins syntaxin 4 and vesicle-associated membrane protein (VAMP) 2/3 also participate in the insulin-stimulated translocation of GLUT4 from intracellular vesicles to the plasma membrane in adipose cells. We now report the molecular cloning and characterization of rat SNAP-23, a ubiquitously expressed homologue of the essential neuronal SNARE protein SNAP-25 (synaptosomal-associated protein of 25 kDa). Rat SNAP-23 is 86% and 98% identical respectively to human and mouse SNAP-23. Southern blot analysis reveals that the rat, mouse and human SNAP-23 genes encode species-specific isoforms of the same protein. Co-immunoprecipitation of syntaxin 4 and SNAP-23 shows association of these two proteins in rat adipose cell plasma membranes, and insulin stimulation does not alter the SNAP-23/syntaxin 4 complex. In addition, we demonstrate for the first time the participation of SNAP-23, along with syntaxin 4 and VAMP2/3, in the formation of 20 S SNARE complexes prepared using rat adipose cell membranes and recombinant α-SNAP and NSF proteins. The stoichiometry of the SNARE complexes formed is essentially identical using membranes from either unstimulated or insulin-stimulated adipose cells. These data demonstrate that rat SNAP-23 associates with syntaxin 4 before insulin stimulation and is present in the SNARE complexes known to mediate the translocation of GLUT4 from intracellular vesicles to the plasma membrane of rat adipose cells.

Key words: GLUT4, insulin, syntaxin, vesicle-associated membrane protein (VAMP).

INTRODUCTION

In muscle, heart and adipose tissue, insulin stimulates the translocation of GLUT4 from an intracellular vesicular pool to the cell surface (reviewed in [1,2]). In the non-insulin-stimulated (basal) state, GLUT4 is mainly stored intracellularly, although under these conditions a low but constant amount of GLUT4 recycling is observed. Insulin dramatically increases the rate of GLUT4 exocytosis, but has little effect on GLUT4 endocytosis, therefore resulting in the rapid appearance of GLUT4 on the plasma membrane following insulin stimulation.

GLUT4 trafficking to the plasma membrane is a regulated exocytic event, and thus the mechanism of insulin-stimulated GLUT4 translocation has been studied based on the basic tenets of the SNARE hypothesis (reviewed in [3–5]). [SNARE stands for SNAP receptor, where SNAP is soluble NSF attachment protein.] This hypothesis predicts that the specific pairing of v- and t-SNAREs (vesicle and target SNARES respectively) is required for vesicle docking and provides specificity to the fusion process. The v-SNARE–t-SNARE complex serves as a binding site for NSF and SNAPs, cytosolic proteins that were originally proposed to facilitate vesicle–target-membrane fusion directly [6]. Recently, however, the role of NSF and SNAPs in the membrane fusion event has come into question ([5,7,8], and references therein), and it is now generally agreed that NSF and SNAP are not directly involved in the fusion reaction itself. Instead, they would act at a pre- or post-fusion step, either to 'prime' the SNARE proteins for docking or to disassemble the SNARE complex once membrane fusion has occurred. In either case, the macromolecular complex of v-SNARE, t-SNARE, SNAPs and NSF can be stabilized in the presence of non-hydrolysable ATP to yield a 20 S SNARE complex that probably represents SNAREs in a pre- or post-fusion state.

Several v- and t-SNAREs have been identified in adipose cells. The v-SNAREs VAMP2 (where VAMP stands for vesicle-associated membrane protein) and VAMP3 (also known as cellubrevin) are found in the GLUT4-containing vesicles [a subfraction of the low-density microsome fraction (LDM)], and the t-SNAREs syntaxin 2 and syntaxin 4 are found in the plasma membrane in rat adipose cells [9,10]. VAMP2 and syntaxin 4 or VAMP3 and syntaxin 4 can form stable complexes together with NSF and α-SNAP [10]. Several observations support the hypothesis that the selective interaction of v-SNAREs, such as VAMP2 and/or VAMP3, with the t-SNARE syntaxin 4 plays a role in the docking and/or fusion of GLUT4-containing vesicles with the plasma membrane. Introduction of the cytosolic domain of syntaxin 4 into 3T3-L1 adipocytes is reported to result in an inhibition of insulin-stimulated GLUT4 translocation [11,12]. In addition, cleavage of VAMP2 and VAMP3 with botulinum neurotoxin B or D, or introduction of the cytosolic domains of

Abbreviations used: HDM, high-density microsomal fraction; LDM, low-density microsomal fraction; PM, plasma membrane fraction; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; v-SNARE, vesicle SNARE; t-SNARE, target SNARE; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosomal-associated protein of 25 kDa.

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The nucleotide sequence data for rat SNAP-23 have been deposited in the GenBank/EMBL/DDBJ Nucleotide Sequence Databases under accession no. AF052596.

VAMP2 and VAMP3, also inhibits insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes [11–15].

In neurons and neuroendocrine cells, another t-SNARE, SNAP-25 (synaptosomal-associated protein of 25 kDa), has been identified in the plasma membrane and shown to interact with the other SNARE proteins [6,16]. SNAP-25 is required for efficient neurotransmitter release from synaptic vesicles and insulin release from pancreatic β-cells [17–19]. A non-neuronal form of SNAP-25 was recently cloned in human lymphocytes, and was called SNAP-23 [20]. SNAP-23 is ubiquitously expressed in human tissues and interacts with VAMPs and syntaxins *in vitro*, suggesting that SNAP-23 could be involved in regulated protein trafficking in non-neuronal cells. A murine isoform of human SNAP-23 (mouse SNAP-23/syndet) was cloned in two independent laboratories [21,22]. Based on the degree of amino acid identity between human SNAP-23 and syndet, Wang et al. [21] proposed that syndet does not represent the murine isoform of human SNAP-23, leading to speculation that the true rodent SNAP-23 isoform remains to be identified.

In the present study, we have isolated a SNAP-23 cDNA clone from a rat adipose cell cDNA library. We report that the rat SNAP-23 protein is highly similar to the mouse and human SNAP-23 proteins. Rat SNAP-23 is localized mainly on the plasma membrane of adipose cells, it does not redistribute upon insulin stimulation, and it is associated with syntaxin 4. Furthermore, we demonstrate for the first time that SNAP-23 participates in the formation of 20 S SNARE complexes together with other v- and t-SNAREs, suggesting that rat SNAP-23 is involved in GLUT4 trafficking in rat adipose cells.

EXPERIMENTAL

cDNA cloning of rat SNAP-23

Approx. 4 g of epididymal adipose tissue from 180–250 g male Sprague–Dawley rats (Charles River) was digested in Trizol (Life Technologies), and total RNA was prepared as described previously [23]. Poly(A)$^+$ RNA was prepared using the QuickPrep Micro mRNA Purification kit, and cDNA was prepared from the poly(A)$^+$ RNA using the First-Strand cDNA Synthesis kit (both kits from Pharmacia Biotech). A portion of rat SNAP-23 cDNA was synthesized from the rat cDNA by PCR amplification using a pair of human SNAP-23 oligonucleotides. These primers amplify a 310 bp DNA fragment corresponding to amino acids 40–140 of human SNAP-23. This DNA fragment was labelled with [^{32}P]dCTP (α) and was used to screen a rat adipose cell λgt11 cDNA library (generously donated by Dr. Alan Kimmel, NIH). The filters were hybridized at 68 °C for 16 h in a buffer containing 0.5 M sodium phosphate, 7 % SDS, 1 mM EDTA and 0.1 % BSA, pH 7.2, and washed for 30 min each in the following buffers: 6 × SSC/0.5 % SDS, 37 °C; 6 × SSC/0.5 % SDS, 42 °C; 6 × SSC/0.5 % SDS, 68 °C; 2 × SSC/0.1 % SDS, 68 °C; and 0.5 × SSC/0.1 % SDS, 68 °C (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). Four independent clones were isolated, and one full-length clone was completely analysed by automated sequence analysis on both strands using an ABI 373 DNA sequencer. The predicted amino acid sequence of rat SNAP-23 was obtained from the nucleotide sequence and aligned with the human and mouse SNAP-23 sequences using MacVector 6.0 (Eastman Kodak).

Southern blotting

Portions of 20 μg of rat, mouse and human genomic DNAs (Clontech Laboratories) were digested to completion with the restriction enzymes *Eco*RI and *Hin*dIII (New England Biolabs). The fragments were resolved on an agarose gel and transferred to a supported nitrocellulose membrane (Micron Separations). The membrane was hybridized at 68 °C for 3 h with a ^{32}P-labelled human SNAP-23 cDNA probe (303 bp; corresponding to amino acids 40–140 of human SNAP-23) in QuickHyb solution (Stratagene), washed for 20 min each in several buffers (6 × SSC/0.5 % SDS, 37 °C; 6 × SSC/0.5 % SDS, 42 °C; 6 × SSC/0.5 % SDS, 68 °C; and twice in 2 × SSC/0.5 % SDS, 68 °C), and exposed to X-ray film. The membrane was then stripped, rehybridized with a ^{32}P-labelled rat SNAP-23 cDNA probe (300 bp; corresponding to amino acids 40–139 of rat SNAP-23), washed as described above, and exposed to a second X-ray film. Finally, the membrane was stripped again, rehybridized with a ^{32}P-labelled mouse SNAP-25 cDNA probe (387 bp; corresponding to amino acids 48–176 of mouse SNAP-25b), washed, and once again exposed to X-ray film. Following each stripping procedure, the membrane was exposed to X-ray film overnight to confirm that all traces of radiolabelled probe had been removed.

Subcellular distribution of SNAP-23

Adipose cells were isolated by collagenase digestion of epididymal fat pads from 180–250 g male Sprague–Dawley rats. Freshly isolated adipose cells were incubated in the absence or presence of 700 nM insulin at 37 °C for 30 min under constant and gentle shaking. Homogenization and subcellular fractionation of the cells to isolate the plasma membrane fraction (PM), the LDM and the high-density microsomal fraction (HDM) were performed as described previously [24], except that 0.12 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin were included in the homogenization buffer. Portions of 20 μg of membrane proteins from each fraction were resolved on SDS/PAGE and analysed for SNARE protein expression by immunoblotting.

Immunoprecipitation and immunoblotting

PMs (50 μg) from basal and insulin-treated rat adipose cells were solubilized with 2 % Triton X-100 in PBS for 30 min at 4 °C and clarified by centrifugation at 48000 ***g*** for 30 min. The clarified solutions were then mixed with anti-SNAP-23 or anti-syntaxin 4 prebound to Protein A–Sepharose. SNAP-23 and syntaxin 4 were immunoprecipitated overnight at 4 °C and washed extensively. The anti-SNAP-23 serum was generated by immunizing rabbits with a peptide sequence corresponding to the 17 C-terminal amino acids of human SNAP-23, as described elsewhere [25]. Western blot analysis was performed as described by Timmers and co-workers [10], using the following antibodies: anti-SNAP-23, anti-GLUT4 (Hoffmann-La Roche), anti-VAMP2, anti-VAMP3 (a gift from Dr. W. Trimble, University of Toronto, Canada), and an affinity-purified anti-syntaxin 4 antibody (a gift from Dr. M. Bennett, University of California at Berkeley, Berkeley, CA, U.S.A.). Labelled proteins were visualized by the enhanced chemiluminescence method (Pierce, Rockford, IL, U.S.A.) and quantified by densitometric scanning (Molecular Dynamics).

Transfection and metabolic labelling

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum. Subconfluent HeLa cells in 10 cm-diam. Petri dishes were transfected with cDNAs encoding human syntaxin 4 and/or human SNAP-23 in the mammalian expression vector pcDNA3 using

the calcium phosphate co-precipitation method. The cells were labelled approx. 36 h after transfection using 0.5 mCi of [^{35}S]methionine for 1 h at 37 °C. Cells were lysed in 1 % Triton X-100 in Tris-buffered saline on ice, and nuclei and other debris were removed by centrifugation at 15000 ***g***. As indicated, cell lysates were incubated alone or mixed together briefly, and specific immunoprecipitations were performed using antibodies bound to Protein A–agarose. The immunoprecipitates were resolved by SDS/PAGE and analysed by fluorography.

Formation of 20 S SNARE complexes

SNARE complexes were formed using Triton X-100-solubilized rat adipose cell PM and LDM together with recombinant α-SNAP and NSF–Myc proteins, as described previously [10]. Briefly, membranes were incubated at 4 °C with 5 μg of Myc-tagged NSF in the absence or presence of 15 μg of α-SNAP in a buffer consisting of 20 mM Tris, pH 7.2, 100 mM KCl, 1 mM ATP and 10 mM EDTA containing 10 μg/ml aprotinin, 10 μg/ml leupeptin and 0.1 mM 4-(2-aminoethyl)benzene-sulphonyl fluoride. SNARE complexes were then adsorbed to a monoclonal anti-Myc antibody prebound to Protein G–Sepharose and washed extensively in binding buffer. SNARE proteins were subsequently eluted by incubation of the NSF–Myc beads with buffer containing 20 mM Tris, pH 7.2, 100 mM KCl, 1 mM ATP and 10 mM $MgCl_2$. Samples were resolved by SDS/PAGE and analysed for SNARE proteins by immunoblotting.

RESULTS

cDNA cloning of rat SNAP-23

To obtain a rat SNAP-23 cDNA probe, we used human SNAP-23 primers to PCR-amplify a 310 bp DNA fragment from rat adipose tissue poly(A)$^+$ RNA. This fragment was used to screen a rat adipose cell cDNA library in order to obtain a full-length putative rat SNAP-23 cDNA. The nucleotide and predicted amino acid sequences of rat SNAP-23 have been deposited in the GenBank Database (accession no. AF052596). The initiator methionine was designated by comparison with the sequence of human and mouse SNAP-23. The rat SNAP-23 cDNA sequence encodes a protein of 210 amino acids with a predicted molecular mass of 23.2 kDa. Rat SNAP-23 does not possess a transmembrane domain, but does contain a cysteine-rich region (amino acids 79–87) that could serve as a potential palmitic acid attachment site. Figure 1 shows the alignment of the predicted amino acid sequences of rat, mouse and human SNAP-23. Overall, rat SNAP-23 is 98 % and 86 % identical respectively to

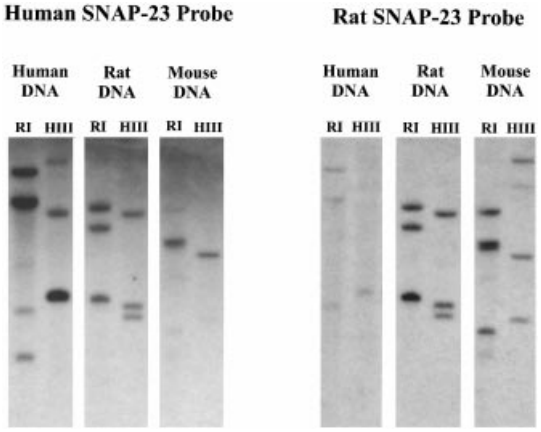

Figure 2 Southern blot analysis of human, rat and mouse genomic DNA with human and rat SNAP-23 probes

Human, rat and mouse genomic DNA was digested with either *Eco*RI (RI) or *Hin*dIII (HIII), and the fragments were resolved on an agarose gel and transferred to a nitrocellulose membrane. The membrane was then probed with a human SNAP-23 probe (left panel) or a rat SNAP-23 probe (right panel) under high-stringency conditions, as described in the Experimental section.

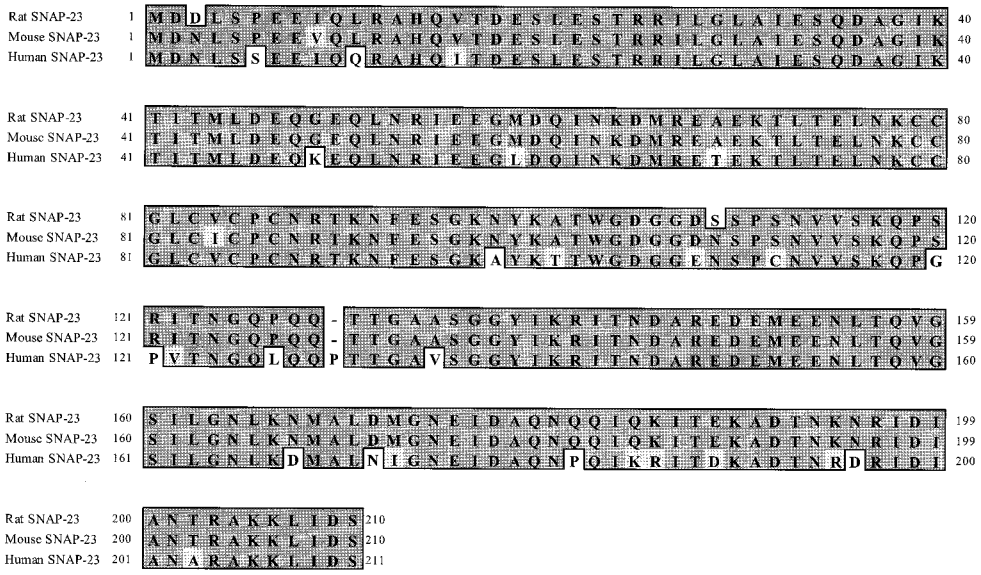

Figure 1 Alignment of the predicted amino acid sequences of rat, mouse and human SNAP-23

The alignment of the rat, mouse and human proteins was optimized using the Clustal method. Identical residues are indicated by the dark grey areas, conserved residues by the light grey areas, and non-conserved residues by the white areas.

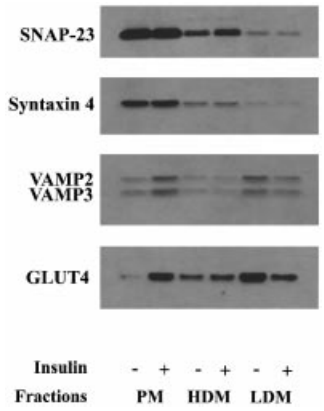

Figure 3 Subcellular distributions of SNARE proteins and GLUT4 in basal and insulin-treated rat adipose cells

Membrane fractions (PM, HDM and LDM) were prepared from basal and insulin-treated adipose cells. Portions of 20 μg of each membrane fraction were analysed by immunoblotting using the indicated antibody. The blot shown is representative of four independent experiments.

mouse SNAP-23 and human SNAP-23, strongly suggesting that rat SNAP-23 is a homologue of these two proteins.

Southern blot analysis of human, rat and mouse SNAP-23 genes

In an attempt to determine the relationship among the genes coding for human, rat and mouse SNAP-23, we analysed the structures of these genes using species-specific DNA probes. Southern blot analysis of genomic DNA from these three species demonstrated that the hybridization patterns are identical if either human SNAP-23 or rat SNAP-23 probes are used in this analysis (Figure 2). In addition, the membrane was also hybridized with a mouse SNAP-25b probe (results not shown). In this case, the hybridization pattern was completely different from that obtained using either SNAP-23 probe, demonstrating that the SNAP-23 probes were not detecting SNAP-25 DNA. These data demonstrate that rat SNAP-23, mouse SNAP-23/syndet and human SNAP-23 encode species-specific isoforms of the same protein.

Subcellular distribution of SNAP-23

The subcellular distribution of SNAP-23 was analysed in membrane fractions prepared from rat adipose cells in the basal and insulin-stimulated states. Figure 3 shows that both SNAP-23 and syntaxin 4 are mainly present in the PM, although they can also be detected to a lesser extent in the HDM and the LDM. Similar results are obtained for the α_1 subunit of the Na^+/K^+-ATPase, a well established PM marker protein in rat adipose cells [24]. Figure 3 also demonstrates that the subcellular localization of SNAP-23 is not changed following insulin stimulation. In contrast, the subcellular distributions of VAMP2, VAMP3 and GLUT4 change from the LDM in the basal state to predominantly the PM following insulin stimulation.

Association of SNAP-23 with syntaxin 4

Since SNAP-23 and syntaxin 4 are both found in the same subcellular fraction, we investigated the possibility that these two t-SNAREs are bound to one another in rat adipose cells *in vivo*. SNAP-23 and syntaxin 4 were immunoprecipitated from Triton

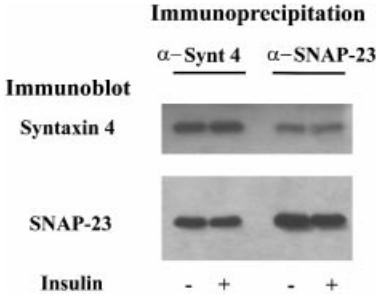

Figure 4 Association of SNAP-23 and syntaxin 4 in rat adipose cell plasma membranes

Syntaxin 4 and SNAP-23 were immunoprecipitated from Triton X-100-solubilized PM from basal (−) and insulin-stimulated (+) adipose cells using an anti-syntaxin 4 (α-Synt 4) antibody and an anti-SNAP-23 antibody respectively. The immunoprecipitates were then resolved by SDS/PAGE, and were analysed for the presence of syntaxin 4 and SNAP-23 by immunoblotting using the same antibodies. The blot shown is representative of three independent experiments.

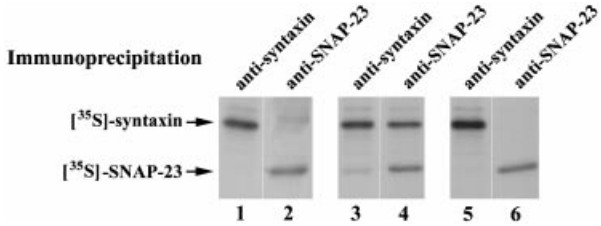

Figure 5 SNAP-23 and syntaxin 4 do not associate after cell lysis

Lysates from [^{35}S]methionine-labelled HeLa cells expressing syntaxin 4 alone (lane 1), SNAP-23 alone (lane 2) or syntaxin 4 and SNAP-23 together (lanes 3 and 4) were subjected to immunoprecipitation using anti-syntaxin 4 (lanes 1 and 3) or anti-SNAP-23 (lanes 2 and 4) antibodies. In addition, lysates from HeLa cells expressing SNAP-23 alone and syntaxin 4 alone were mixed (lanes 5 and 6) and subjected to immunoprecipitation using anti-syntaxin 4 (lane 5) or anti-SNAP-23 (lanes 6) antibodies. The immunoprecipitates were analysed by SDS/PAGE and fluorography. The electrophoretic mobilities of [^{35}S]syntaxin 4 and [^{35}S]SNAP-23 are indicated.

X-100-solubilized PMs obtained from basal and insulin-treated rat adipose cells using anti-SNAP-23 and anti-syntaxin 4 antibodies respectively. SDS/PAGE and Western blotting revealed that approximately half of the total immunoprecipitable pool of syntaxin 4 was associated with SNAP-23, as determined by densitometry (Figure 4, upper panel). Similarly, approximately half of the total immunoprecipitable pool of SNAP-23 was associated with syntaxin 4 (Figure 4, lower panel). Neither SNAP-23 nor syntaxin 4 was immunoprecipitated when a pre-immune serum was used (results not shown). In agreement with the results of the subcellular fractionation studies, the stable association between SNAP-23 and syntaxin 4 in rat adipose cells was not affected by insulin treatment.

To ensure that the complex-formation observed between syntaxin 4 and SNAP-23 actually occurred in cells and was not a post-solubilization artifact, we radiolabelled HeLa cells expressing syntaxin 4 alone, SNAP-23 alone or syntaxin 4 and SNAP-23 together, and subjected cell lysates to immunoprecipitation analyses. In agreement with the results obtained in rat adipose cells, immunoprecipitates of HeLa cells expressing both syntaxin 4 and SNAP-23 revealed efficient co-precipitation of syntaxin 4 with the SNAP-23 antibody and vice versa (Figure 5, lanes 3 and 4). In contrast, when lysates of HeLa cells

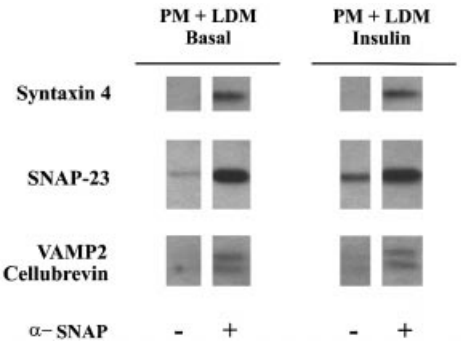

Figure 6 Incorporation of SNARE proteins from rat adipose cell membranes into 20 S SNARE complexes

SNARE complexes were formed from PM and LDM prepared from basal or insulin-stimulated rat adipose cells by incubating the solubilized membranes with recombinant NSF–Myc in the absence (−) or presence (+) of α-SNAP. The complexes were bound to a monoclonal anti-Myc antibody prebound to Protein G–Sepharose in the presence of non-hydrolysable ATP. The SNARE proteins were specifically eluted with MgATP and analysed by immunoblotting using specific antibodies. The blot shown is representative of two independent experiments.

expressing syntaxin 4 alone and SNAP-23 alone were mixed prior to immunoprecipitation, no binary complex-formation was detected (Figure 5, lanes 5 and 6). As anticipated, analysis of HeLa cells expressing syntaxin 4 alone or SNAP-23 alone also failed to reveal complex assembly (Figure 5, lanes 1 and 2), demonstrating that a significant degree of syntaxin 4/SNAP-23 complex-formation did not occur following cell lysis. Taken together with the results of Figure 4, these data demonstrate that SNAP-23 and syntaxin 4 form stable complexes in the PM of rat adipose cells *in vivo*.

SNAP-23 is present in the 20 S SNARE complex

The t-SNAREs syntaxin and SNAP-25, and the v-SNARE VAMP, form a stable complex that is able to bind NSF in an α-SNAP-dependent manner [6]. To determine if the homologous SNAREs from rat adipose cells behave in a similar way, 20 S SNARE complexes were bound to NSF–Myc beads using membrane fractions prepared from basal or insulin-stimulated rat adipose cells, in either the absence or the presence of recombinant α-SNAP (Figure 6). SNARE complexes were bound to NSF–Myc beads under conditions preventing ATP hydrolysis and then specifically eluted with MgATP. The inclusion of α-SNAP in the mixture markedly increased the formation of 20 S SNARE complexes and the subsequent elution of syntaxin 4, SNAP-23 and VAMPs. The small amount of 20 S SNARE complexes detected in the absence of recombinant α-SNAP can readily be explained by the presence of small amounts of endogenous α-SNAP in the membrane fractions [26]. Figure 6 also demonstrates that the composition of the 20 S SNARE complex was not different when complexes were prepared using membranes from basal or insulin-stimulated adipose cells. This result is important, since it shows that, like the association of SNAP-23 and syntaxin 4, the SNARE protein content of the 20 S complex is not significantly affected by insulin signalling in adipose cells.

DISCUSSION

The SNARE hypothesis proposed by Söllner et al. [6] predicts that transport vesicle docking with target membranes requires the interaction of vesicle-associated VAMPs with the target-membrane-associated proteins syntaxin and SNAP-25 to form a macromolecular protein complex. One key component of this complex, SNAP-25, is found almost exclusively in the brain, whereas syntaxins and VAMPs are expressed ubiquitously, suggesting the existence of a non-neuronal homologue of SNAP-25. This ubiquitously expressed homologue, termed SNAP-23, has been identified in both human [20] and murine [21,22] tissues. Although human and mouse SNAP-23 share a high level of amino acid identity, the lack of complete amino acid conservation between these SNAP-23 isoforms has led to speculation that mouse SNAP-23/syndet is not the murine isoform of human SNAP-23 [21]. In the present study, we have cloned and partially characterized rat SNAP-23. This protein is 86% identical with human SNAP-23 and 98% identical with mouse SNAP-23/syndet. Furthermore, Southern blot analysis of rat, mouse and human genomic DNA using either rat or human SNAP-23 probes yields essentially identical hybridization patterns, demonstrating that rat, mouse and human SNAP-23 genes encode species-specific isoforms of the same protein.

Rat SNAP-23 contains two major domain structures, which strongly suggests that this protein is a SNAP-25 homologue. Hydrophobicity analysis predicts that, like human and mouse SNAP-23, rat SNAP-23 has no transmembrane domain (results not shown). However, all three of these SNAP-23 homologues possess a cysteine-rich region that contains possible sites for palmitoylation. If the entire cysteine cluster in SNAP-25 is deleted, or if two or more cysteine residues in the cysteine-rich region of mouse SNAP-23 are deleted, then these proteins are found in the cytosol rather than associated with the plasma membrane [27,28]. In the case of SNAP-25, it has been demonstrated that this deletion prevents palmitoylation [27]. Since rat SNAP-23 has the same five cysteine residues as human and mouse SNAP-23, it is very likely that the rat protein will also be membrane-associated through palmitoylation of some of these cysteine residues. In addition to the putative palmitoylation domain of SNAP-23, both SNAP-25 and SNAP-23 have a high probability of forming coiled-coil structures [21,29,30]. These coiled-coils have been shown to be involved in protein–protein interactions necessary for the association of SNAP-25 with syntaxin and VAMP [29,30]. Rat SNAP-23 also possesses regions proposed to form coiled-coils, suggesting that this protein also associates with syntaxins and VAMPs via coiled-coil interactions.

In agreement with the subcellular localization of mouse SNAP-23 in 3T3-L1 adipocytes [21,22,31] and of human SNAP-23 in epithelial cells [25,32], rat SNAP-23 is found primarily in the PM in rat adipose cells. Small amounts of SNAP-23 can also be found in the intracellular membrane fractions (HDM and LDM), but these are likely to represent contamination of HDM and LDM with PM [24]. Alternatively, it is also possible that a small fraction of SNAP-23 is trapped during membrane internalization or that SNAP-23 is associated with syntaxin isoforms present on intracellular membranes. In agreement with this latter hypothesis, SNAP-23 has been detected on intracellular membranes in HepG2 cells and MDCK cells [25,33], and SNAP-23 binds to syntaxin 11, a syntaxin isoform present in small amounts on intracellular compartments [33a].

SNAP-25 interacts with syntaxin and VAMP to form a stable ternary complex *in vitro* and *in vivo*, and this complex disassembles in the presence of α-SNAP, NSF and MgATP [6,34–36]. SNAP-23 has been clearly shown to bind to syntaxin and VAMP *in vitro* [20,22], but the binding of SNAP-23 to SNARE proteins in non-transfected cells remains to be clarified. Foster et al. [37] reported that syntaxin 4 is present in an anti-SNAP-23 immunoprecipitate, but, surprisingly, SNAP-23 was

not detected in an anti-syntaxin 4 immunoprecipitate from 3T3-L1 adipocytes. In the present study, however, we demonstrate that SNAP-23 and syntaxin 4 are efficiently co-precipitated from basal rat adipose cell PM, regardless of the combination of antibodies used for immunoprecipitation and Western blotting. Furthermore, insulin stimulation does not alter the stoichiometry of the SNAP-23/syntaxin 4 complex. Since the functional neuronal t-SNARE exists as a complex of syntaxin with SNAP-25, one possible effect of insulin in adipose cells could have been to enhance the association of syntaxin 4 with SNAP-23 following insulin stimulation, thereby generating a SNAP-23/syntaxin 4 complex only after insulin stimulation. We did not observe such an alteration in the association of SNAP-23 with syntaxin 4 in the present studies, strongly suggesting that these proteins exist as a pre-formed 't-SNARE' complex on the plasma membrane.

SNAP-25 is present in 20 S SNARE complexes containing NSF, SNAPs, syntaxin and VAMP from a variety of brain tissues [6,16]. Using non-neuronal cells, Timmers et al. [10] have demonstrated the *in vitro* formation of SNARE complexes containing α-SNAP and NSF using basal adipose cell PM (containing syntaxin 4) and LDM (containing VAMP2 and VAMP3). However, the presence of a SNAP-25 homologue in these SNARE complexes has been assumed, but has not been shown directly. We now show for the first time that SNAP-23 is present in SNARE complexes formed using solubilized rat adipose cell membranes, and that the association and dissociation properties of SNAP-23 in the SNARE complex appear to be identical with those of SNAP-25 in the neuronal membrane-derived SNARE complex. We have also detected SNAP-23 in 20 S SNARE complexes formed using solubilized mouse kidney membranes (P. A. Roche, unpublished work), demonstrating that the presence of SNAP-23 in SNARE complexes is not restricted to adipose cells. Finally, we have demonstrated that the composition and stoichiometry of the 20 S SNARE complex is not affected by insulin treatment of adipose cells. Timmers et al. [10] demonstrated that SNARE complexes isolated from basal and insulin-treated adipose cells are similar, in that they contain comparable amounts of syntaxin 4 and VAMP 2/3. However, since only half of the total pool of syntaxin 4 is associated with SNAP-23 in adipose cells, it was formally possible that 20 S SNARE complexes formed from insulin-treated cells would contain syntaxin 4 and VAMP2/3, but would lack SNAP-23, thereby resulting in distinct SNARE complexes in pre- and post-stimulated cells. Since insulin stimulation did not alter the 20 S SNARE complex, it is most likely that only syntaxin 4/SNAP-23 complexes (and not free syntaxin 4) are present in SNARE complexes from adipose cells.

Sadoul et al. [38] have demonstrated that transfected SNAP-23 can replace SNAP-25 in the insulin release process in a pancreatic β-cell line, therefore providing the first evidence for a functional role for SNAP-23 in regulated exocytosis. During the preparation of the present paper, Rea et al. [39] reported that introduction of either anti-SNAP-23 antibodies or a synthetic SNAP-23 peptide significantly inhibited insulin-stimulated GLUT4 translocation, highlighting the importance of SNAP-23 in adipose cell biology. In the present paper, we extend this observation by demonstrating that rat SNAP-23 is present in 20 S SNARE complexes prepared from adipose cell membranes containing syntaxin 4, SNAP-23, VAMP2/3, α-SNAP and NSF. Taken together with the data of Sadoul et al. [38], it is likely that SNAP-23 is functioning in a manner analogous to that of SNAP-25 in neuronal and neuroendocrine cells. Our working hypothesis regarding the role of SNARE proteins in GLUT4 translocation is that, subsequent to the insulin-stimulated signal for the translocation of GLUT4-containing vesicles to the plasma membrane, the v-SNAREs VAMP2/3 bind to a pre-existing complex of syntaxin 4 and SNAP-23 in the plasma membrane. Whether or not the SNAREs are 'primed' on either the vesicle membrane or the plasma membrane by α-SNAP and NSF is not known, although we would predict that priming of the SNAREs for fusion also occurs in adipose cell membranes, as has been observed in other systems (reviewed in [5,40]). Furthermore, our data demonstrate that insulin does not alter the composition of the complex. Since insulin stimulation leads to the translocation of VAMP from intracellular vesicles to the plasma membrane, the effect of insulin on SNARE complex-formation appears to be in the translocation event and not the formation of the 20 S SNARE complex. It remains to be determined what molecular machinery is responsible for the initial recruitment of GLUT4-containing vesicles to the plasma membrane. Clearly, further work is required to elucidate the complete array of proteins required for GLUT4 trafficking to the plasma membrane, although it is likely that SNARE complexes containing SNAP-23, syntaxin 4 and VAMP2/3 are essential components of this process.

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